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Antigenic Diversity of Hepatitis B Virus Strains of Genotype F in Amerindians and Other Population Groups from Venezuela

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The adw4 subtype of hepatitis B virus (HBV) belongs to a unique genomic group (genotype F) representing the original HBV strains from the New World. Data regarding the prevalence of this subtype among HBV carriers in South America are, however, scarce, and those concerning HBV genotype F are based on only a few samples from Latin America. In this study, serum samples were obtained from 141 hepatitis B surface antigen (HBsAg) carriers from Amerindians and urban populations from Venezuela. The HBsAg subtype was identified with monoclonal antibodies in 105 samples, and the HBV genotype was identified by reverse-phase hybridization with DNA fragments in 58 samples. The adw4 subtype was highly prevalent in the population studied (75%); among the Amerindians, the prevalence was 97%. The adw2 subtype was also present (10%), while other subtypes (ayw3 and ayw4) were only occasionally found. The HBV subtype was associated with the expected genotype in most cases (80%), and thus genotype F was highly prevalent. Sequencing of viral strains that gave genotypes unpredicted by the HBsAg subtyping confirmed seven of them as belonging to not previously described genotype-subtype associations: namely, adw2 and ayw4 within genotype F.

Hepatitis B virus (HBV) strains isolated worldwide have been classified into six genomic groups deduced from genome comparisons and indicated as HBV genotypes A to F (10, 17). Nine serological groups, called hepatitis B surface antigen (HBsAg) subtypes, have also been defined based on discriminating sera and have been designated adw2, adw4, adr, adrq−, ayw1, ayw2, ayw3, ayw4, and ayr (5). It has been shown that each of the known HBsAg subtypes may belong to either one or several genotypes, but in such cases, the genotype involved, rather than the subtype, is more likely to correlate with the geographical origin of the strain (14).

Data regarding the prevalence of HBsAg subtypes among carriers from South America are scarce (5, 8), and those concerning the HBV genotypes in this area have been based on results from only a few samples from Latin America (1, 4, 11, 12, 14, 16, 25). However these results do suggest that (i) the adw4 subtype defines a unique genomic group (genotype F), (ii) this HBV genotype is the prevalent infectious agent in the original human populations of America, and (iii) it may represent the first split from the human hepadnaviral ancestor (11, 15, 16).

To provide further data regarding the genomic characteristics and antigenic variability of the HBV strains from South America, a study was carried out involving the identification of HBV subtypes and genotypes in a large number of HBV strains obtained from Amerindians and other populations from Venezuela.

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MATERIALS AND METHODS

The study comprised 141 serum samples from HBsAg carriers from the following populations: (i) Amerindians from Western Venezuela (Barí and Yukpa [$n = 23$], with prevalences for HBV infection of 11.1 and 7.1%, respectively [3]); (ii) Amerindians from South Venezuela (Yanomamis from the Orinoco Basin [$n = 12$], a group from a region in which HBV infection is highly endemic; 7.4% prevalence of HBsAg [26, 27]); (iii) hemodialysis patients from units with a high prevalence of HBV and hepatitis C virus (HCV) infection in Caracas ($n = 24$) (19) and in Maracaibo ($n = 40$); (iv) patients with chronic hepatitis from Maracaibo (where HBsAg positivity was determined over a period of at least 6 months and histological evidence of chronic hepatitis was observed upon biopsy [$n = 19$]); (v) hemophiliacs from Maracaibo ($n = 12$); (vi) blood donors from Caracas ($n = 9$), where HBsAg prevalence has been determined as 0.71% (18); and (vii) pregnant women from Puerto La Cruz (0.4% prevalence of HBsAg) (7).

The HBsAg subtype was determined on the basis of the reactivity pattern by enzyme immunoassay (EIA) with a panel of five monoclonal antibodies (3C3, 2D11, 3D9, 3A5, and 3E2) as described elsewhere (24). HBV genotypes were determined with DNA fragments amplified by PCR with a reverse-phase hybridization assay with genotype-specific probes (line probe assay [LiPA]) (InnoLiPA HBV; Innogenetics SA, Ghent, Belgium). HBV genotypes were determined in the pre-S1 and HBsAg regions. The HBV LiPA genotyping technology (22) is comparable to the test described for HCV genotyping (23). Primers used for the amplification were HBPr 134 (outer sense, 5′ TGCTGCTATGCCTCATCTTC 3′), HBPr 135 [outer antisense, 5′ CA(G/A)AGACAAAAGAAAATTGG 3′], HBPr 75 (nested sense, 5′ Bio-CAAGGTATGTTGCCCGTTTGTCC 3′), and HBPr 94 [nested antisense, 5′ Bio-GGTA(A/T)AAAGGGACTCA(A/C)GATG 3′].

In 18 samples, a region of the HBsAg gene corresponding to amino acids 81 to 180 was amplified by PCR and sequenced. The HBV DNA in proteinase K-treated serum was amplified with primers hep3 and hep33. The amplified product was purified and used as a template in a sequencing reaction with hep3 as sequencing primer (13). In three other samples, the region of the gene corresponding to the major external region of HBsAg was amplified between codons 109 and 206 with primers HBPr 75 and HNPr 94. This PCR fragment was purified to remove unincorporated nucleotides and primers and was further analyzed with an ABI 373 sequencer by dideoxy chain terminator chemistry with the same primers for sequencing. In all of these samples, the genotype for each strain was assessed on the basis of sharing at least 12 of 13 amino acid substitutions in this region known to be conserved within genotypes (see Table 3). The HBsAg subtype was deduced from the amino acid substitutions at positions 122, 127, and 160 (13).

TABLE 1. HBsAg subtypes according to monoclonal typing among carriers from different population groups from Venezuela

Population group	No. (%) with HBsAg subtype:						
	ad	adw2	adw4	adr[a]	ayw3	ayw4	Total
Amerindians							
West	0	1	22	0	0	0	23
South	0	0	6	0	0	0	6
Caracas							
Blood donors	0	0	8	0	0	0	8
Hemodialysis patients	1	2	9	0	1	5	18
Maracaibo							
Chronic patients	4	7	2	1	0	0	14
Hemodialysis patients	3	1	30	0	0	0	34
Hemophiliacs	0	0	0	0	0	0	0
Puerto La Cruz (pregnant women)	0	0	2	0	0	0	2
Total	8 (7.6)	11 (10)	79 (75)	1 (1)	1 (1)	5 (4.8)	105[b] (100)

[a] This strain was revealed upon sequencing to be subtype adw2 (Table 3).
[b] A total of 141 samples were subtyped. Only 105 of them had an HBsAg concentration high enough to be efficiently typed by monoclonal antibodies.

Statistical differences were evaluated by the chi-square test with Yate's correction and by Fisher's exact test when a value was less than 5, according to a computerized Epi Info program, version 5.01b (Centers for Disease Control and Prevention, Atlanta, Ga.).

RESULTS

In order to assess the HBV genetic variability present in Venezuela, the HBsAg subtype was analyzed for 141 HBV-infected patients and the HBV genotyping was performed for 58 samples. From the 141 serum samples, 105 could be subtyped by monoclonal EIA (Table 1). The adw4 subtype was highly prevalent in all of the population groups studied, except for the hemodialysis patients from Caracas and chronic patients from Maracaibo, in whom a wide range of subtypes were circulating, including both ayw3 and ayw4. Among Amerindians, adw4 accounted for 97% of the infections. The adw2 subtype was detected among patients with chronic hepatitis and patients undergoing hemodialysis. Subtype ayw4 was significantly associated with a single hemodialysis unit compared to its prevalence in other population groups ($P < 0.001$).

Out of the 58 genotyped samples, a total of 47 were infected with HBV genotype F strains, either alone or in a mixture with other genotypes (6 samples). These mixed infections were only found among hemodialysis patients. Genotypes A and D were detected in 10 samples, and genotype B was detected in one patient (Table 2). In 46 cases, both the HBsAg subtype and the

TABLE 2. HBV genotypes and HBsAg subtypes, assigned by rapid methods, among 58 HBV carriers from Venezuela

Population	No. with HBsAg subtype of HBV genotype:												
	A				B (adr)	D (NA)	F				Mixed with F[b]		
	ad	adw2	adw4	NA[a]			adw2	adw4	ayw4	NA	adw4	ayw3	NA
Amerindians													
West	0	0	1[c]	1	0	0	0	7	0	0	0	0	0
South	0	0	0	0	0	2	0	5	0	1	0	0	0
Caracas													
Blood donors	0	0	0	0	0	0	0	5	0	0	0	0	0
Hemodialysis patients	0	1	0	0	0	0	1[c]	8	5[c]	2	1	1	3
Maracaibo													
Chronic patients	1	5	0	0	1[c]	0	0	0	0	0	0	0	0
Hemodialysis patients	0	0	0	0	0	0	0	3	0	0	1	0	0
Hemophiliacs	0	0	0	0	0	0	0	0	0	3	0	0	0
Puerto La Cruz (pregnant women)	0	0	0	0	0	0	0	2	0	0	0	0	0
Total ($n = 59$)	1	6	1[c]	1	1[c]	2	1[c]	29	5[c]	6	2	1	3

[a] NA, not assignable. A total of 12 genotyped samples could not be typed by monoclonal antibody subtyping because of insufficient HBsAg concentration.
[b] Mixed infections were observed as more than one band in the genotyping LiPA.
[c] Unexpected association of subtype and genotype.

TABLE 3. Genotype- and subtype-specific amino acid substitutions between residues 81 and 180 of the primary structure of HBsAg

Genotype-subtype combination	Genotype- or subtype-specific substitution at amino acid:															
	85	110	114	122[a]	126	127[a]	131	134	140	143	158	159	160[a]	161	168	178
A/adw2	F	I	T	K	T	P	N	F	T	T	F	A	K	Y	V	P
B/adw2	C	I	S	K	T	P	T	F	T	T[b]	F	A	K	Y	V	P
C/adr	F	L	S	K	I/S	P	T	F	T	S	F	A	R	F	V	P
C/adw2	F	L	S	K	T	P	T	F	T	S	F	A	K	F	V	P
D/ayw3	F	I	S	R	T	T	T	Y	T	S	F	G	K	F	A	P
D/ayw2	F	I	S	R	T	P	T	Y	T	S	F	G	K	F	A	P
E/ayw4	F	I	S	R	T	L	T	F	S	S	F	G	K	F	A	P
F/adw2	F	L	T	K	T	P	T	F	S	S[c]	L	G	K	Y	A	Q
F/adw4	F	L	T	K	T	L	T	F	S	S[c]	L	G[d]	K	Y	A	Q
F/ayw4	F	L	T	R	T	L	T	F	S	S	L	G	K	Y	A	Q

[a] Subtype-specific substitutions.
[b] HBV strain in sample from chronic patient 22506 (B/adw2) had an M at position 143.
[c] HBV strains in samples from hemodialysis patients D29 and D36 (F/adw4) had an L at position 143.
[d] HBV strain in sample from Amerindian 755 (A/adw2) had a V at position 159.

HBV genotype were available for comparison. In all samples but eight (83%), the subtype determined by EIA was associated with the expected genotype determined by LiPA (Table 2).

A total of 19 samples were also studied by sequence analysis, and the genotype and subtype were assigned as shown in Table 3. Genotype-subtype combination F/adw4 was confirmed in six serum samples, and A/adw2 was confirmed in five others. One unexpected genotype-subtype association (subject 755 [A/adw4], a West Amerindian) was found upon sequencing of A/adw2. One unexpected subtype in this population group (subject 22506 [B/adr], a chronic patient) was revealed upon sequencing to belong to subtype adw2. A methionine at position 143 for the isolate from subject 22506 and a valine at position 159 for the isolate from subject 755 might be responsible for the mistyping by monoclonal antibodies (Table 3). Moreover, one adw2 sample and all five ayw4 samples showed the unexpected HBV genotype F (Table 2). Sequence analysis confirmed the HBsAg subtype and the genotype predicted in all of these specimens. An additional specimen (hemodialysis patient D5 [genotype F]), which could not be subtyped by EIA because of insufficient HBsAg, was revealed upon sequencing to belong to the same genotype-subtype group of F/ayw4.

DISCUSSION

In agreement with previous reports regarding the HBsAg subtypes in South America (5–7), this study showed that the adw4 subtype was highly prevalent among HBV carriers from Venezuela, being almost unique among the Amerindians. As expected, most of the adw4 strains were grouped into genotype F, and this genotype was the most prevalent among the samples studied. These findings support prior conclusions regarding the American origin of this genotype (1) and its correlation with the adw4 subtype (11–13, 15). Most adw2 strains were grouped in genotype A, and this is likely to reflect its North American or European origin (14, 25). Infections with multiple genotypes were detected in seven cases of infection, and these mixed infections always involved genotype F. All of these cases were found among hemodialysis patients, who are considered high-risk patients for multiple infections by parenterally transmitted viruses. Such mixed infections in high-risk patients have previously been documented for HCV genotypes (9), particularly in this group of patients (20). Slightly different results between serotyping and genotyping were observed concerning these mixed infections, but this discrepancy can be explained by the higher sensitivity of the genotypic amplification procedures (PCR amplification of few viral copies) compared to that of the serotyping technology (requiring larger amounts of HBsAg protein).

Besides the high correlation found between HBsAg subtypes and HBV genotypes, eight samples from this study were classified into HBV genomic groups which were not predicted by the subtyping (Table 2). Such discrepancies involved samples from four of the subtypes found in the study, but especially concerned samples from the ayw4 subtype that were all recognized as genotype F. The confirmation of both subtype ayw4 and genotype F after sequencing of six strains and adw2 subtype and genotype F in one strain suggests that two previously unrecognized geno-antigenic groups of HBV strain (subtypes ayw4 and adw2, genotype F) exist in South America. These new associations were only found among hemodialysis patients from the same unit from Caracas, where nosocomial transmission of HBV could be playing a role in viral dissemination as it seems to for HCV (19). Indeed, the distribution of HBV variants in hemodialysis patients from Caracas was significantly different from that observed in other patient groups, suggesting that nosocomial transmission might explain this cluster of F/ayw4 in this setting. However, the presence of genotype F/ayw4 cannot exclusively be attributed to an outbreak in a hemodialysis unit from Caracas, because this new association has recently been found in other Latin American countries (2). In contrast, more European-like HBV strains were found circulating among chronic patients from Maracaibo. This situation is probably due to the European immigration that has always been significant in Venezuela. Interestingly, strains of HBV from the old-world lineages were also observed in the Amerindians tested (Tables 1 and 2), suggesting that these strains had been introduced even in the more isolated communities from Venezuela.

Genotype F of HBV has been defined on the basis of sequences obtained from strains from France, Alaska, Colombia, and Brazil (11–14, 16). Its full characterization is likely to require the study of additional samples from other populations. On the other hand, the HBV strains from the ayw4 subtype have been previously classified into two different genotypes, genotype E in Africa and a single genotype D strain from the United States, MS-2 (14). The new genotype-subtype associations found in this study corroborate the genetic diversity of the ayw4 subtype and suggest an antigenic diversity inside genotype F, which would now include adw2 and ayw4 in addition to adw4. Table 4 summarizes the present knowledge

TABLE 4. Distribution of HBV genotypes and HBsAg subtypes across the world

HBV genotype	HBsAg	Geographical distribution[a]
A	adw2	Europe, North America, Africa
	ayw1	Africa
B	adw2	Far East
	ayw1	Far East
C	adrq−	Pacific
	adr/ayr	Far East
	adw	Japan, Indonesia
	adr	Far East, Pacific
D	ayw4	United States[b]
	ayw2/ayw3	Worldwide
E	ayw4	Africa
F	adw2	South America[c]
	adw4	Polynesia, Alaska, Central and South America
	ayw4	South America[c]

[a] See reference 10.
[b] Strain MS-2.
[c] Association shown in this work.

about the genotype-subtype associations of HBV, including the novel data reported here.

Genotyping of HBV strains might also give an explanation for some pathogenic aspects of HBV infection which are likely to show peculiarities in some geographical areas (14). The ability to be transmitted vertically, oncogenic potential, and susceptibility to vaccine-induced immunity could be different in some HBV genotypes, and these differences might explain the predominance of mother-to-child transmission in the spread of HBV in the Far East (21), the geographical variations in the incidence of the HBV-associated liver cancer, or the failure of vaccine-induced immunity to prevent HBV infection reported in Africa (6). More severe cases of HBV coinfection with hepatitis delta virus have also been described in South America, and a phenomenon of coevolution of HBV genotype F with hepatitis delta virus genotype III has been suggested and might be responsible for this severe form of disease (4). Therefore, the high predominance of genotype F strains found in this study warrants future investigations of these aspects of HBV infection in South America.

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